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Title: **METHOD FOR THE RAPID DETERMINATION OF BACTERIA**

The invention relates to the detection,
identification or determination of bacteria in samples in
general and in particular in clinical samples such as·
blood, urine, saliva, cerebrospinial fluid, faeces, pus
5   and tissue that are taken from patients that are possibly
infected with a, as yet unknown, possibly pathogenic
bacterium, or during follow-up diagnostic testing to for
example evaluate therapeutic measures that have been taken
so far to treat the disease.

10   Traditional methods to determine or identify bacteria
in general start with a Gram-stain, which is well known in
the art. Such a stain can be performed on a sample
immediately after sampling or, when not enough bacteria
are present, after a short period of culturing of the
15   sample. In general, four types of bacteria are found after
Gram-staining; Gram-negative rods and cocci and Gram-
positive rods and cocci. However, such a Gram-stain can
only in very exceptional cases provide the clinician with
the knowledge required to provide accurate therapy.

20   Examples of Gram-negative rods in clinical samples
are *Enterobacter*, *Klebsiella*, *Salmonella*, *Escherichia*,
Proteus and *Pseudomonas species*, of Gram-negative cocci
are *Neisseria species*. Gram-positive rods that may be
found in clinical samples are *Bacillus species*, of Gram-
25   positive cocci are *Enterococcus*, *Streptoccus* and
Staphylococcus species. Some of these, such as *Streptoccus*
and *Staphylococcus* can easily be further determined or
distinguished from each other by their morphological
characteristics. *Streptococci* (and *Enterococci*) are
30   characterised by chain-like character of cocci that are

Streptoco__ species cannot be distingu__ed by morphology alone. However, such relatively rough taxonomic distinction on a genus level cannot be considered satisfactory for clinical purposes and consequently

5   further identification is required to establish proper medication. For example in the case of *Staphylococcus*, these bacteria need to be further distinguished based on their coagulase positive (*S. aureus*) or coagulase negative (*S. haemolyticus* and others) character because these two

10   groups require different antibiotic therapy.

In general, the exact species involved is determined by culturing techniques. To fully determine the species of a bacterium present in a clinical sample the following steps are in general required:

15   - (1) Pre-culturing of the sample in order to amplify the number of bacteria to a level above the lower detection limits of step (2).

- (2) Culturing on selective and non-selective media.

These traditional methods are time consuming. On

20   average, a regular diagnostic procedure takes at least a few hours (minimally 2) of pre-culturing followed by minimally 24 hours of culturing on selective and non-selective media. This implies that it takes at least 26 hours before the clinician obtains a diagnosis on which he

25   or she can select appropriate antibiotics or base other further treatment.

This latency-period between the sampling of a patient and the final diagnosis in most cases is critical for the treatment and the speed-of-recovery of the patient. During

30   this latency-period a patient is in general treated with broad-spectrum antibiotics. The antibiotic of choice is mainly determined by the 'clinical eye' of the clinician.

By selecting a broad-spectrum antibiotic, such therapies are in general _____ of _____ _____ _____

is affected also. This side-effect heavily decreases the
patients defence against microbial invaders from the
environment. Especially the lowering of the colonisation
threshold of the gastro-intestinal tract may cause severe

5   overgrowth by e.g. yeasts and fungi. The resulting
secondary infection, or super infection, in septicaemic
patients who already suffer from a decreased immunity
often leads to life-threatening situations.

Apart from the serious danger to the patient's

10   health, wide-spectrum antibiotic therapy poses another
threat. The repeated exposure of indigenous bacteria to
antibiotics enhances the emergence of resistance against
such an antibiotic. Especially when a resistance-gene is
encoded on a plasmid, other (potential pathogenic)

15   bacterial species may become resistant after the uptake of
the plasmid. This latter scenario is considered to be a
major problem in hospital epidemiology. It is therefor of
paramount therapeutic and epidemiological importance to
speed up the methodological procedures in the diagnosis of

20   blood samples from for example septicaemic patients to be
able to select specific antibiotic therapy designed for
the specific pathogen found, thereby refraining from using
broad-spectrum antibiotics.

Present techniques other than culturing, albeit in

25   general specific when beforehand knowledge exists about
the species involved, cannot be used with samples
containing uncharacterised species, and do thus not fit
the acute needs when speedy diagnosis is needed on
uncharacterised patient material. In general, these

30   methods are also too slow to meet the needs of the
clinician in providing care to his or her patients. Most,
for example, require isolation of nucleic acid, or
amplification of nucleic acid, or both, before the actual
testing can be performed. Also in those cases where

identify ⬤ pathogen and rapidly select ⬤ e antibiotic against which the pathogen is not resistant. To do this, the micro-organism would again have to be cultured, to determine its resistance pattern, thereby again

5   introducing a lag in diagnosis.

In short, there is a need for fast and reliable diagnosis of bacteria, present in for example clinical samples that may replace or add to the currently used culturing techniques.

10

The invention provides a method for determining, detecting or identifying a bacterium suspected of being present in a sample comprising

     a) testing said sample by Gram-staining and

15   b) testing said sample with a probe according to an in situ hybridisation protocol selected on the basis of the outcome of said Gram-staining.

Rapid techniques for detecting bacteria and other bacteria in general are known. For example, in situ

20   hybridisation is a well known technique, however, in general it has only been applied in specialised laboratories as a tool to detect and quantify the relative abundance of bacteria that are difficult to culture using traditional methodology or as a tool to quantify for

25   example growth kinetics of already known bacteria in culture.

In short, in in situ hybridisation, nucleic acid probes, labelled with a reporter molecule such as an enzyme or a fluorescing substance, are reacted with

30   specific nucleic acid sequences found specifically and preferably solely in the bacteria under study, which for this purpose has been permeabilised to let the probe enter the organism. As a target sequence nucleic acids of

23S ribosomal RNA, mitochondrial RNA, messenger RNA and
nuclear DNA.

      In situ hybridisation has never been successfully
applied for rapid detection of bacteria in clinical
5   samples because the presently used *in situ* hybridisation
techniques are too inaccurate and too slow to give an
advantage over traditional culturing.

      First of all, hybridisation requires permeabilisation
of the pathogen, and until now no generally applicable
10 permeabilisation protocols have been developed that allow
sufficient but restricted lysis of many or all of a broad
range of unidentified bacteria. In general, mild
permeabilisation leaves many bacteria (such as
Staphylococcus spec.) inaccessible for subsequent
15 hybridisation with probes, whereas rigorous
permeabilisation often fully lyses most bacteria, thereby
foregoing the possibility to detect them all together.

      In addition, current protocols are in general time-
consuming multi-step procedures; hybridisation often
20 requires minimally 24 hours, thereby giving no relief to
the needs of the clinician who is only helped with
accurate and speedy diagnosis. Furthermore, they mostly
require beforehand knowledge about the genus or even
species involved in order to select appropriate probes;
25 having such beforehand knowledge is clearly not the case
in the event of a patient having an unidentified
infection. Also, the present, already inappropriate
hybridisation techniques do not allow to gather
information on the response against antibiotics of the
30 bacterium involved.

      The invention provides a fast and reliable method for
diagnosis, detection and/or determination of bacteria
which may be present in a sample. Such a sample may be of
various origin, it is for instance possible to apply a

from a (contaminated) bacterial culture, drinking water or food suspected to be contaminated with a bacterium.

In a preferred embodiment the invention provides a method to detect or identify a bacterium suspected of being present in a clinical sample. Herein, the term "clinical sample" comprises a sample obtained or derived from an animal, preferably a mammal, more preferably a human being. Such a sample may be sampled or tested because a bacterial infection or disease is suspected. Such a sample can be of various origin, such as blood, serum, white blood cells, cerebrospinal fluid, synovial fluid, tissue, biopsies, urine, saliva, faeces, and others. In a preferred embodiment the invention provides a method wherein said sample is mammalian blood, preferably being derived from a human.

A sample can be a primary sample or it can be a secondary or sub-sample which is derived from a primary sample by diluting, splitting or culturing it one or more times. Diluting allows determining the relative abundance of a bacterium in a sample, thereby thus providing a method allowing not only qualitative but also quantitative determination of a bacterium. A sample can be tested directly after it has been obtained or after it has been stored, for example by cooling or freezing and secondary or sub-samples can be tested in parallel or subsequent from each other.

The invention provides a method comprising determining by Gram-staining the Gram-positive or Gram-negative and rod or coccus type of bacterium in a clinical sample and further testing said sample according to an in situ hybridisation protocol selected on the basis of the outcome of said Gram-staining. A primary advantage of a method according to the invention is the speed with which analyses can now be performed. Th

protocols for example, Gram-positive Streptococci can now
be determined from within about 30-60 minutes, Gram-
negative rods from within about 45-90 minutes, if needed,
whereas traditional protocols often need a working day or
5   more. On top of that, often a first indication, or even a
definitive selection, of a preferred antibiotic for
therapy can be given, based on the results of the testing.

A preferred embodiment of the invention is a method
for the detection or identification of bacteria in a
10   clinical sample of blood of patients who are suspected to
suffer from a septicaemia. In a preferred embodiment a
method provided by the invention makes use of labeled
probes, such as fluorescently labeled single strain DNA-,
RNA- or PNA-probes, directed against specific target
15   sequences on for example the ribosomal RNA of the target
bacterium present in the sample.

The invention provides a method wherein classical
Gram-staining indicates the presence of a Gram-negative or
Gram-positive bacterium in said sample, further comprising
20   determining the rod or coccus character of said bacterium,
thereby establishing the subsequent testing protocol.

When a Gram-negative bacterium is of the rod type, the
invention provides a method further comprising hybridising
said sample with at least one probe selected from a group
25   of probes capable of hybridising with nucleic acid found
in *Escherichia coli*, in *Klebsiella pneumoniae*, in
Klebsiella oxytoca, in *Serratia marcescens*, in
Enterobacter aerogenes, in *Enterobacter cloacae*, in
Proteus vulgaris, in *Proteus mirabilis*, in *Salmonella*
30   *typhi*, in *Pseudomonas aeruginosa*.

Furthermore, the invention provides a method wherein
said character is of the Gram-negative coccus type,
further comprising subjecting said sample to treatment
with ...

comprising determining the rod or coccus character of said
bacterium, and when said Gram-positve character is of the
rod type, further comprising subjecting said sample to
treatment with a lysis buffer comprising lysozyme and/or
5   Proteinase K.

In addition, when said character is of the Gram-
positive coccus type, a method is provided further
comprising determining a chain-like or clump-like
character of said bacteria before a hybridisation protocol
10 is selected. When before mentioned character is chain-
like, a method provided by the invention is further
comprising subjecting said sample to treatment with a
lysis buffer comprising lysozyme, and further comprising
hybridising said sample with at least one probe selected
15 from a group of probes capable of hybridising with nucleic
acid found in *Enterococcus faecalis*, in *Streptococcus
pneumoniae*, in *Streptococcus mitis*, in *Streptococcus
viridans*, in *Streptococcus sanguis*, in *Enterococcus
faecium*.

20   In addition, a method is provided wherein said character
is clump-like, further comprising subjecting said sample
to treatment with a lysis buffer comprising lysostaphin
and or Proteinase K, further comprising hybridising said
sample with at least one probe selected from a group of
25 probes capable of hybridising with nucleic acid found in
Staphylococcus aureus, in *Staphylococcus haemolyticus*, in
Staphylococcus saprophyticus.

Probes used in a method as provided by the invention
can be directed against various target nucleic acid
30 molecules found in a bacterium which can be used are for
example ribosomal RNA, mitochondrial RNA, plasmid DNA,
messenger RNA and nuclear DNA. It is also possible to
select as target molecules nucleic acid from the above
discussed antibiotic resistance genes, which can be for

In a ●ferred embodiment, a method ●vided by the invention uses as a target for in situ hybridisation a (16S) ribosomal RNA molecule. In a particular embodiment of the invention said probe is having no more than five,

5   preferably no more than two mismatches with a probe selected of a group composed of probes having a sequence GCCTGCCAGTTTCGAATG or GTAGCCCTACTCGTAAGG or GAGCAAAGGTATTAACTTTACTCCC or GTTAGCCGTCCCTTTCTGG or TTATCCCCTCTGATGGG or AGAGAAGCAAGCTTCTCGTCCG or

10   GCCACTCCTCTTTTTCCGG or GCTAATGCAGCGCGGATCC or CCGAAGGGGAAGGCTCTA or AGAGAAGCAAGCTTCTCGTCCGTT, each selected in relation to a method as provided by the invention or in relation to congruent antibiotic sensitivity of a bacterium recognised by said probe.

     In addition, a method is provided by the invention that is further comprising hybridising said sample with at least one positive control probe capable of hybridising with nucleic acid found in a majority of bacterial species and/or with at least one negative control probe not being

20   capable of hybridising with nucleic acid found in a majority of bacterial species. Preferably said majority comprises at least 90% of bacterial species, especially with those species found in general with possibly infected (septicaemic) patients. A method as provided by the

25   invention is even more specific and/or sensitive when at least 95%, preferably at least 99% of said species is reactive with said positive control probe or no more than 5%, preferably no more than 1% is reactive with said negative control probe.

30   Such a positive or negative control probe as provided by the invention is given in the experimental part, in general said positive control probe comprises no more than five mismatches with a probe with the sequence GCTGCCTCCCGTAGGAGT and/or said negative control probe

Furthermore, the invention provides a method with
additional value to the clinician in that in said method
a probe is selected for its reactivity with one or a
group of bacterial genera and/or (sub)species having
5   congruent susceptibility to antibiotic treatment. Such a
probe detecting or identifying a bacterium in a sample,
preferably a clinical sample, is capable of hybridising
with nucleic acid found in a group of bacterial genera
and/or species or subspecies such as found with
10   Staphylococcus and many other bacteria having congruent
susceptibility to antibiotic treatment.

In a preferred embodiment of the invention, such a
probe is having no more than five, preferably no more than
two mismatches with a probe selected of a group composed
15   of probes having a sequence GCCTGCCAGTTTCGAATG or
GAGCAAAGGTATTAACTTTACTCCC (i.e. reactive with bacteria
for which amoxycillin treatment is most likely effective)
or GTAGCCCTACTCGTAAGG (cephalosporin treatment) or
GTTAGCCGTCCCTTTCTGG (piperacillin and/or aminoglycoside)
20   or TTATCCCCTCTGATGGG or GCCACTCCTCTTTTTCCGG (amoxycillin)
or GCTAATGCAGCGCGGATCC or CCGAAGGGGAAGGCTCTA
(vancomycin) or AGAGAAGCAAGCTTCTCGTCCGTT or
AGAGAAGCAAGCTTCTCGTCCG (flucloxacillin).

In a much preferred embodiment of the invention a
25   one-step procedure is used for both binding target
bacteria (in the sample) to a microscopic slide and fixing
intracellular structures. In the experimental part,
various lysis buffers and fixating technique are provided
that utilise such a one-step procedure.
30   Furthermore, the invention provides a diagnostic test
kit comprising means for detecting or identifying a
bacterium suspected of being present in a sample using a
method according to the invention or using a probe
according to the invention.

Instructi⬤ for a method comprising in ⬤u hybridisation
may be added. Optionally, said probes, which can be common
nucleic acid or peptide nucleic acid probes, are linked to
reporter molecules such as direct fluorescent labels.
5   Other reporter molecules, such as enzymes or radioactive
labels are also known

In addition, said kit may comprise one or more of the
necessary buffer solutions, such as lysis buffer or
hybridisation, optionally in ready made form, or for
10   example cover slips and reaction vials. Said kit may fully
comprise sets of probes reactive with a wide gamut of
(pathogenic) bacteria, optionally characterised by
reactivity with bacteria of congruent antibiotic
susceptibility, or may comprise sets of probes
15   specifically directed against bacteria of Gram-positve of
-negative, rod, coccus or chain- or clump-like character.

Such a kit may also comprise probes specifically
reactive with antibiotic resistance genes, providing a
positive identification of least applicable antibiotic
20   treatment.

The invention is further explained in the
experimental part of the description which is not limiting
the invention.

25   Experimental part

An example of a set of probes specific for the
detection of pathogenic bacteria and an example of a new
protocol for high-speed in situ hybridisation are
30   presented. The methodology described here is for example
used for both a preliminary screening of samples from
septicaemic patients or as a full substitute on the basis
of which therapeutic decisions are made. The invention
thus provides a rapid and reliable method for determining

Components

A set of fluorescently labeled oligonucleotide probes
designed to hybridise specifically with a group of
pathogenic bacteria (i.e. genus-specific probe) or with
one specific pathogen (i.e. species-specific probe) or
with bacteria with congruent susceptiblity or resistance
to antibiotics.

A protocol for fast in situ hybridization of bacteria
present in samples of blood collected from septicaemic
patients, using the said probes.

Oligonucleotide probes designed to hybridize specifically
with a group of pathogenic bacteria.

In a particular embodiment of the invention a method
provided by the invention is exemplified by making use of
16S rRNA target molecule because a large databank
containing 16S rRNA-sequences exists and is freely
accessible via the Internet. Labeled probes form an
essential part in in situ hybridizations. The present
invention provides a set of probes which have been
designed in an unexpected novel manner i.e. not based on
normal taxonomic principles but rather on their
pathological significance. The group of probes which apply
to this particular embodiment of the invention have been
designed in such a way that they span group(s) of bacteria
which are clustered on the basis of presumed congruent
sensitivity to antimicrobial agents. Positive
identification thus yields direct therapeutic information.
Grouping bacteria on the basis of their presumed
antibiotic susceptibility results in groups of bacteria

on the criteria of presumed antibiotic susceptibility patterns is much faster over classical culturing methods that still suffer from fenotypic variability induced by environmental factors. The probes are preferably labelled
5 with enzymatic or fluorescent labels. Current fluorescent labels which are applicable in this invention are:

1) Direct fluorescent labels:
- Fluorescein-isothiocyanate (FITC)
10 - Tetramethylrhodamine-5-isothiocyanate (TRITC)
- Texasred"
- 5(6)-carboxyfluorescein-N-hydroxysuccimide-ester (FLUOS")
- 7-amino-4-methylcoumarin-3 acetic acid (AMCA")
15 - Phycoerythrin
- Indocarbocyanine dyes such as Cy3", Cy5" and C7"
- Any other direct fluorescent label
2) Indirect fluorescent labels:
- Enzymes such as alkaline phosphatase or
20 horseradishperoxidase either attached directly or via a
C6-thiol linker and used in combination with
chemiluminescent substrates like AMPPD (3-
('spiroadmantane)-4-methoxy-4-(3'-phosphoryloxy)-phenyl-
1,2-dioxethane) or fluorescence generating substrates.
25 - Digoxigenin (DIG) in combination with anti-DIG
antibodies labeled with:
- gold particles
- fluorescent labels
- Enzymes such as alkaline phosphatase or horseradish
30 peroxidase, optionally in combination with
chemiluminescent substrates like AMPPD (3-
('spiroadmantane)-4-methoxy-4-(3'-phospho- ryloxy)-phenyl-
1,2 dioxethane) or fluorescence generating substrates.
- Biotin in combination with streptavidin

- Dinitrophenyl as hapten in combination with appropriate antibodies and labeled just like the anti-DIG antibodies

- Any other indirect fluorescent or enzymatic label

Fluorescent labels allow direct microscopic analysis preferably combined with image analysis. For the detection of fluorescent oligonucleotide probes hybridised to ribosomal RNA of the target bacterium, photography can be applied. However quantitation by this method is hampered by the absence of objective criteria by means of which discrimination between hybridized and non-hybridized cells can be performed. Therefore for objective evaluation of probe-specificity, an image analysis system is employed which allows fluorimetrical reading of individual bacterial cells.

A protocol for fast *in situ* hybridization of bacteria present in samples of blood

Protocols for the detection of rRNA *in situ* typically utilise both a lytic reagent for permeabilisation of the bacterial cell wall and fixatives to preserve structural and molecular integrity of cellular components. However, the results of such hybridizations are highly dependent on the type, concentration and incubation-time of both the lytic reagent and the fixative. Component 2 of the invention consists of a protocol for *in situ* hybridization in which both permeabilisation and fixation have been optimized for a subsequent hybridization of maximally 2 hours. For this protocol it was important to ensure that the hybridization procedure used was applicable to a wide variety of unidentified bacteria. Differentiated use of lytic reagents could only be based on information obtained from direct Gram staining of the blood culture but a

A optimal lytic reagent can be chosen on the basis of the gram-stain of the pathogen present in the sample of blood. This procedure of differentiated permeabilisation is novel to regular protocols for in situ hybridisation in
5   which the permeabilisation is always dedicated to the permeabilisation of one or a defined group of target bacteria. In this new procedure a very wide array of unidentified bacteria can sufficiently be permeabilised without destruction of intracellular structures.

10   A one-step procedure is used for both binding target bacteria (in the sample) to the microscopic slide and fixing intracellular structures. Procedures presented in the current scientific literature all use multi-step protocols for binding, fixing and dehydration of the
15   bacterial cells in order to condition them for optimal hybridisation.

The hybridisation time is shortened to 2 hours. Regular protocols for in situ hybridisation utilise a hybridisation time of minimally 24 hours, rendering them
20   useless for rapid diagnostic applications.

The invention also provides kits for carrying out the rapid detection of bacteria in blood samples according to the invention. Such a kit will usually comprise at least a probe or probes and optionally other reagents such as
25   components for hybridisation-fluid, washing-fluid and permeabilisation-fluid.

Such a kit may be applied in a routine bacteriology laboratory or in a bedside environment, both as a fast screening method or as a full substitute for classical
30   identification methods.

Examples of probe design and development

are found in blood from septicaemic patients. In addition
each probe hybridises with a species or a cluster of
bacteria which share congruent (but often not identical)
antibiotic sensitivity patterns

ID	Sequence (5'-3')[1]	Region[2]	Specificity[3]
A	GCTCCCTCCCGTAGGAGT	V2	Bacterial Kingdom
B	ACTCCTACGGGAGGCAGC	n.d	no matches
C	GCCTGCCAGTTTCGAATG	V2	Salmonella spp, Klebsiella spp, Enterobacter spp.
D	GTAGCCCTACTCGTAAGG	V7	K. oxytoca, S. marcescens, Enterobacter spp, Proteus spp
E	GAGCAAAGGTATTAACTTTACTCCC	V3	E. coli
F	TTATCCCCCTCTGATGGG	V2	E. faecalis
G	CCTAATGCAGCGCGGATCC	V2	S. aureus, S. haemolyticus
H	CCGAAGGGAAGGCTCTA	V6	S. aureus, S. saprophyticus
I	AGAGAAGCAAGCTTCTCGTCCG	V1	Streptococcus spp.
J	GTTAGCCGTCCCTTTCTGG	V3	P. aeruginosa
K	AGAGAAGCAAGCTTCTCGTCCGTT	V2	S. aureus
L	GCCACTCCTCTTTTTCCGG	??	Enterococcus faecium

[1] Each probe optionally contains an FITC-label at the 5'-
end

[2] The variable region on the 16S rRNA where the target-
sequence of the probe is positioned.

[3] The species or genus which rRNA contains a match with the
sequence of the probe.

Protocol example.

A newly devised protocol for fast in-situ hybridization of
pathogens in blood from septicaemic patients. This version
consists of a step-wise version which can directly be used
in a laboratory environment.

1   Collect a sample of blood from a patient using a
vacuum sealed culture bottle.

2   Place the culture bottle in the pre-culturing
machine (e.g. BactAlert, Organon Teknika, Durham, NC
27704) to monitor the growth of the pathogen. On-line

3   After bacterial growth in a sample blood has been detected, perform a Gram-stain and take out the culture bottle and collect 1 ml. of blood from the bottle using a syringe.

5   4   Using the syringe, put +/- 0.1 ml of this sample on a degreased glass slide. And streak out using a slide of glass.

5   Dry the slide for 5 minutes on a hotplate (for example of approximately 50°C).

10   6   Fix during 5 min. in ethanol(96%):formaldehyde(37%) (9:1.)

7   Dry the slide for 5 minutes on a hotplate. (Slides can be stored for several months if kept at room temperature in a dry chamber)

15   8   Permeabilise **Streptococci** 20 min at 25 C with lysozyme (1 g/l)

9   Permeabilise **Staphylococci** 20 min at 25 C with Lysostaphin (100 units/ml)

10   Rinse the slide with (demineralised) water for 5

20   minutes

11   Dry the slide for 5 minutes on a hotplate.

12   Pipet hybridisation buffer(+SDS)-probe mix ([probe]=10ng/μl). Cover with a coverslip.

13   Hybridize 2 hours (for example at 48°C).

25   14   Rinse 5 min using hybridisation buffer(-SDS).

15   Mount the slide with a coverslip.

16   Evaluate the slide.

phosphate buffered saline

30   - 8 g/l NaCl

- 0.2 g/l KCl

- 1.44 g/l Na$_2$HPO$_4$

- 0.24 g/l KH$_2$PO$_4$

- adjust to pH 7.4

Hybridisation buffer (+SDS)

- 900 ml Milli-Q water

- 52.6 g NaCl

- 2.52 g Tris (hydroxymethyl)-aminomethane

5 - adjust to pH 7.5

- add 90 ml water.

- sterilize 15 minutes

- 10 ml SDS (10%) stock

10 hybridisation buffer (-SDS)

- 900 ml Milli-Q water

- 52,6 g NaCl

- 2,52 g Tris

- adjust to pH 7.5

15 - 100 ml Milli-Q water

- sterilize 15 minutes

hybridisation buffer-probe mix

- 10 ng/ul of lyophilized probe in hybridisation buffer

20

lysozyme buffer

- 1,2 g Tris (=100mM)

- 1,86 g EDTA (=50mM)

- add 100 ml of milli-Q

25 - adjust to pH 7.5

- 0,05 to 0,2 g lysozyme

Lysostaphin buffer

- 1,2 g Tris (=100mM)

30 - 1,86 g EDTA (=50mM)

- add 100 ml of milli-Q

- adjust to pH 7.5

- 0,05 to 0,2 g lysostaphin

- dilute a lysostaphin stock 1000 ng/ml in milli-Q

Optionally, to lyozyme buffer or lyostaphin buffer 0,05
to 0,2 mg/ml Proteinase K is added.

5   ethanol-formaldehyde (90:10)
     - 1 ml formaldehyde 37%
     - 9 ml ethanol 96%

     Validation of probe specificity
10   Specificity of probes was tested against the complete RDA-
     database (http://rdpwww.life.uiuc.edu:80/rdphome.html) of
     15 august 1996 using the CheckProbe command and was
     considered sufficient if a no more than five, preferably
     no more than two mismatches were observed. Furthermore, to
15   determine whether the probes could reach their specific
     target sequence, a reference collection of 20 of the most
     predominant bacteria in sepsis were hybridised using both
     the protocol and the probes mentioned here above. The
     result of this validation is listed in table 2. As can be
20   read from this table all probes yield a satisfying
     hybridisation profile. Using the group-probes C and D it
     is possible to distinguish between: four groups of gram-
     negative rods:
     C-positive and D-positive: Klebsiella oxytoca,
25   Enterobacter cloacae and Enterobacter aerogenes
     C-positive and D-negative: Klebsiella pneumoniae and
     Salmonella typhi
     C-negative and D-positive: Serratia marcescens and Proteus
     vulgaris
30   C-negative and D-negative: Proteus mirabilis.
     For Escherichia coli and Pseudomonas aeruginosa two
     species-specific probes (E and J) have been designed and
     validated. These probes are optionally included because
     both Escherichia and Pseudomonas are notorious pathogen

notorious ⬤ thogen. Probe I is a *genus-* ⬤ ific probe
which can be used in conjunction with probe F because
Streptococci and Enterococci share the same morphology,
while they require different antimicrobial treatment.

5   Using both probes G and H, 4 separate species of
Staphylococci can be distinguished:
G-positive and H-positive: Staphylococcus aureus
G-positive and H-negative: Staphylococcus haemolyticus
G-negative and H-positive: Staphylococcus saprophyticus

10   G-negative and H-negative: Staphylococcus epidermidis
Probe K is a *species*-specific-probe for Staphylococcus
aureus and can be used to support the results obtained by
probes G and H.

15

Table 2.

Bacterium	A	B	C	D	E	F	G	H	I	J	K
Escherichia coli	■				■						
Klebsiella pneumoniae	■		■								
Klebsiella oxytoca	■		■	■							
Serratia marcescens	■			■							
Enterobacter aerogenes	■		■	■							
Enterobacter cloacae	■		■	■							
Proteus vulgaris	■		■								
Proteus mirabilis	■										
Salmonella typhi	■		■								
Pseudomonas aeruginosa	■								■		
Enterococcus faecalis	■					■					
Enterococcus faecium	■										
Streptococcus pneumoniae	■							■			
Streptococcus mitis	■							■			
Streptococcus viridans	■							■			
Streptococcus sanguis	■										
Staphylococcus haemolyticus	■						■				
Staphylococcus aureus	■						■				■
Staphylococcus epidermidis	■										
Staphylococcus saprophyticus	■							■			

Legend: Probecoding see table 1, gray=positive
hybridization, white=no hybridization

5

Testing a method in whole-blood samples.
Preliminary testing of a new method in 50 whole blood
samples which were found positive upon pre-culturing
yielded a correlation of 96% between a method described
10   here and the classical culturing method which was also
applied to each of the 50 samples.
However, the results of a method described here could be
obtained within 3 hours while culturing results took a
mean analysis-time of 32 hours.

15

Further practical application
Septicemia is a pathological condition in which viable and
multiplying bacteria may be present in the bloodstream.
This condition may occur after trauma or surgery
20   (especially of the visceral organs), immunosuppression and
obstetrical complications. It is a potential life-
threatening condition and appropriate information on the

is routinely carried out by selective culturing of blood,
which has previously been incubated on a general medium in
a blood culture system. Currently, three continuous-
reading, automated and computed blood culture systems are

5   available in clinical microbiology today: the BacTec 9240
(Becton Dickinson Instruments), the BacT/Alert (BTA,
Organon Teknika) and the Extra Sensing Power (Difco
Laboratories). All of these machines measure the
production-rate of a bacterium specific metabolite in the

10 culture-bottle containing the patient's blood supplemented
with general nutrient broth. Subsequently if bacterial
metabolic activity is detected, the positive blood culture
sample is plated on appropriate selective media for
further analysis. Microbial identification, taking the

15 positive blood culture as a starting point, takes 24 h to
72 h to complete. Reduction of the analysis time may
result in reduction of the use of broad-spectrum
antibiotics as the genus or species of a pathogen gives an
indirect indication of the most appropriate antibiotic.

20 Subsequently, this may result in a lower frequency of
emergence of resistance against broad-spectrum
antibiotics. Furthermore, it may result in lower cost
because suppressive empiric therapy may be substituted by
tailored and less expensive antibiotics with a smaller

25 spectrum. Several methods for rapid detection of pathogens
in human blood have been described previously, most of
them using the polymerase chain reaction or fluorescently
labeled probes. Although these methods are fast and
accurate, routine bacteriological analysis still heavily

30 relies on classical culturing technique. It was therefore
decided that for a molecular biological method to be
successfully implemented in routine bacteriology it should
be fast (maximally 3-4 h) and very easy-to-use (e.g. as
complex as the preparation of a blood smear).

oligonucleotide probes, was developed and validated. The
probes described in this further practical application
section comprise of single strain oligonucleotides
labelled with fluorescein iso-thiocyanate at the 5'end;
5   complementary to a genus- or species-specific sequence on
the 16S- or the 23S ribosomal RNA of the target-organism.

Materials and methods

10   *Blood samples*

During the time of this study a total of 182 blood samples
which tested positive in the BactAlert-blood culture
machine were processed simultaneously, using both whole-
15   cell hybridization and accepted culturing methods.

Culturing

Microorganisms cultivated from positive blood cultures
20   were identified by using the API-testsystem (BioMerieux,
France) or by using standard microbiological methods.

Probes

25   The characteristics of the probes used in this study are
listed in Table 3. All probes consist of a single stranded
oligonucleotide sequence covalently linked with
fluorescein iso-thiocyanate at the 5'-end. Probes were
synthesized by EuroGentec BV (Maastricht, The Netherlands)

30

Table 3.   ●igonucleotide probes used fo● ●ybridization of
some pathogens normally detected in blood from septic
patients

Probe*	Sequence (5'-->3')	Target(s)	Preferred antibiotic**
EUB	GCTGCCTCCCGTAGGAGT	Bacterial Kingdom	n.a.***
nor-EUB	ACTCCTACGGGAGGCAGC	negative control	n.a.
STREP	GTTAGCCGTCCCTTTCTGG	Streptococcus spp.	Penicillin G
EFAEC	TTATCCCCCTCTGATGGC	Enterococcus faecalis	Amoxycillin
EFAEM	GCCACTCCTCTTTTTCCGG	Enterococcus faecium	Vancomycin
STAUR	AGAGAAGCAAGCTTCTCGTCCG	Staphylococcus aureus	Flucloxacillin
CNS	CGACGGCTAGCTCCAAATGGTTACT	Coagulase-negative Staphylococci	Vancomycin
ECOLI	GCAAAGGTATTAACTTTACTCCC	Escherichia coli	Amoxycillin
PSEUDAER	GGACGTTATCCCCCACTAT	Pseudomonas aeruginosa	Piperacillin+ aminoglycoside
ENTBAC	CATGAATCACAAAGTGGTAAGCGCC	Enterobacterium spp	2nd generation cephalosporin

5

*   All probes consist of single strain DNA covalently linked with
fluorescein iso-thyocyanate at the 5'-end. The *E. coli*-specific probe
is directed against 23S rRNA, the other probes are directed against
the 16S rRNA. Probe-nomenclature consists of mnemonics instead of a
10   formal nomenclature-system for reasons of convenience.
**   The antibiotic of first choice
***   not applicable
****   Limited choice from all available therapeutics. On the basis of
the local epidemiological situations other therapeutics may prevail
15

Whole cell hybridization

After gramstaining a streak out preparation of a positive
blood culture, a choice was made on the subsequent
20   permeabilization-protocol and a set of appropriate probes
(Table 3). Grampositive streptococci were permeabilized by
incubating the fixed slide in a permeabilization-buffer (1
mg/ml lysozyme) during 5 min, gram-positive staphylococci

during 20 ▓▓ n. Gram-negative rods were n▓ permeabilized.
Other gram-morphologies were not considered in this
section because of the low incidence of these groups of
bacteria in septicemia. From a positive blood culture

5   fifteen µl was pipetted on a glass slide and subsequently
streaked out. After air-drying the slide, the cells on the
slide were fixed in a 4% formaldehyde-solution in pure
ethanol. After permeabilization, the cells on the slide
were hybridized at 50°C. Gram-negative rods were

10   hybridized during 45 min, gram-positive staphylococci were
hybridized during 2 h and gram-positive streptococci were
hybridized during 5 min. Per gram-type a different set of
probes was chosen (see Table 4).

Table 4 Performance of the method

Application-criterion*	Probe	Target organism	n**	r***
Each assay	EUB	All bacteria	182	1.00
	non-EUB	negative control	182	1.00
Gram-positive chains	STREP	Streptococcus spp.	20	1.00
	EFAEC	Enterococcus faecalis	10	1.00
	EFAEM	Enterococcus faecium	7	1.00
Gram-positive clumps	STAUR	Staphylococcus Aureus	13	1.00****
	CNS	Coagulase-negative Staphylococci	73	1.00
Gram-negative rods	ECOLI	Escherichia coli	23	1.00
	PSEUDAER	Pseudomonas aeruginosa	4	1.00
	ENTBAC	Enerobacterium spp.	23	1.00

* The application criterion is the micromorphology of the pathogen in
5   the initial gram-stain which is made after the BactAlert blood culture
machine has detected microbolic activity in the bloodsample
** n=number of strains positively identified by culturing
*** r=correlation coefficient. This is the number of matching
identifications between FISH and traditional culturing divided by the
10 total number of assays
**** Comprises of 66% of the total number of samples tested. Other
samples showed no signal with the positive control probe

However, a probe (i.e. EUB-probe) positive for almost all
15 bacteria and the reverse complementary probe (i.e. non-EUB
probe) were included as a positive and negative control
respectively, irrespective of the gram-type. Prior to use,
probes were diluted to a concentration of 10 ng/ml in
hybridization buffer (20 mM Tris-HCl, 0.9M NaCl, 0.1% SDS,
20 pH 7.2). After hybridization, the slides were rinsed
during 10 min at 50°C in washing buffer (20 mM Tris-HCl,
0.9M NaCl, 0.1% SDS, pH 7.2) and mounted with VectaShield
(Vector Laboratories, Burlinghame, USA). Immediately
hereafter, the slides were evaluated using an
25 epifluorescence microscope.

The results of this section show that identification using whole-cell hybridization dramatically increases the speed of the diagnosis. In Figure 1 a typical example of the microscopic image obtained after hybridizing a blood

5   sample obtained from a patient suffering from *Streptococcus pneumoniae* sepsis using the STREP-probe is shown. Using the described protocol, a clear-cut positive signal was obtained. Repeated microscopic evaluation by different observers confirmed the unambiguity of the

10   interpretation of the images obtained by this method. In Table 4, the results of the study are listed. The observation that all strains hybridize positively with the EUB-probe indicates that the hybridization protocol is applicable for whole cell hybridization of the bacterial

15   species and genera tested in this study. The negative results obtained with the non-EUB probe indicate the absence of aspecific interaction between the probe and constituents of the cellular matrix. The speed of diagnosis (after the sample is positive in the BactAlert

20   blood culture machine) varies between 25 min (streptococci/enterococci) and 2 h (staphylococci), while routine bacteriological determination would take at least 24 h to 48 h. The advantage to the patient is obvious because, as can be read from Table 3, the clinician is

25   able to start appropriate antimicrobial therapy within the working day instead of after 24 h to 48 h. Being able to choose the most appropriate antibiotic also diminishes the need for broad-spectrum antibiotics therewith indirectly lowering the incidence of antibiotic-resistance. In table

30   4 the results obtained in this section are mentioned.

Broader application of the product

In its current form the product can be used for four o

other clinical samples. Experiments using the current
product (i.e. probes and protocol) in another type of
clinical sample have been carried out. The sample-types
were: liquor and ascites. Results indicate that
5 application of the product in these samples is perfectly
well possible. Future application include:

- New probes for other relevant species/genera
- Other types of clinical samples like: sputum, pus,
10 urine, where generally non-septicaemic bacteria, such as
Legionella pneumoniae can be found, using a method
according to the invention.

Advantages of whole-cell hybridization

15

- Rapid diagnosis enabling the clinician to easily choose
the appropriate antibiotic
- Less use of broad spectrum antibiotics, therewith
lowering the incidence of antibiotic resistance
20 - Cheap, a typical FISH-analysis is about 50% cheaper than
a traditional culturing-based analysis.
- Easy to perform. A FISH-based analysis requires less
actions and less hands-on time than a gram stain

Lay-out of kits

There are many possibilities for possible kit formats,
several are listed below.
One main kit for 100 Tests (1 test is 1 positive control,
30 1 negative control and one unknown) consisting of all kits
1-4 listed below including a detailed protocol. Or the
kits listed below alone or in a combination.

Kit 1 (the control kit):

- Lyophilized positive control probe (such as EUB or
probe(s) functionally related thereto)
5   - Lyophilized negative control probe (such as non-EUB or
probe(s) functionally related thereto)
- Lyophilized hybridization buffer (20 mM Tris-HCl, 0.9M
NaCl, 0.1% SDS, pH 7.2)

10 Kit 2 for gram-negative samples:

- Lyophilized probe such as ECOLI, PSEUDAER, ENTBAC or
probe(s) functionally related thereto
Lyophilized hybridization buffer (20 mM Tris-HCl, 0.9M
15 NaCl, 0.1% SDS, pH 7.2)

Kit 3 for gram-positive streptococci-like bacteria:

- Lyophilized probes such as STREP, EFAEC, EFAEM or
20 probe(s) functionally related thereto
- Lyophilized hybridization buffer (20 mM Tris-HCl, 0.9M
NaCL, 0.1% SDS, pH 7.2) with 1 mg/ml hen-eggwhite
lysozyme

25 Kit 4 for staphylococci-like bacteria:

- Lyophilized probes such as STAUR, CNS or probe(s)
functionally related thereto
- Lyophilized hybridization buffer (20 mM Tris-HCl, 0.9M
30 NaCl, 0.1% SDS, pH 7.2) with 10 units/ml lysostaphin

Basic labeling of probes is with fluorescein iso-thio
cyanate. Alternatively kits may contain probes with other
fluorescent labels e.g.

with different fluorescent labels for simultaneous detection of different targets with one test. We have successfully detected streptococci and enterococci in one sample in this way. In Fig 2 an example of this type of

5   application is shown. Here, a mixed infection of morphologically indistinguishable gram-positive streptococcus-like bacteria are successfully hybridized with both the STREP-probe (FITC-label) and the EFAEC-probe (Cy3-label). Other combinations of the above kits may also

10 be provided as one kit for a specific application.

Legends

Figure 1: Cells of *Streptococcus pneumoniae* show intense
bright fluorescence after 5 min. of incubation with the
5   STREP-probe at 50°C. Magnification = 10x100, fluorochrome
= fluorescein iso-thiocyanate

Figure 2: Mixed infection with *E. faecalis* and *S.*
pneumoniae hybridized simultaneously with both the STREP-
10   probe (FITC-label) and the EFAECAL-probe (Cy3-label). Also
a fluorescent DNA/RNA stain (DAPI) has been applied to
detect all nucleic acid.